8. Brief statement of working hypothesis: Genetic and biochemical analyses of temperature sensitive and conditional lethal mutants of RNA-containing viruses which replicate in eukaryotic cells have proven to be extremely powerful methods for the elucidation of the molecular basis of the replication and of the disease-causing properties of such viruses. AMV is a typical member of the C-type RNA-containing tumor viruses which have been demonstrated to be associated with such human neoplastic diseases as rhabdomyosarcomas, breast cancers, various sarcomas and lymphomas, and leukemia. No temperature sensitive or conditional lethal mutants of AMV have ever been isolated. This is due to the fact that no suitable screening or assay system was available. This difficulty has now been overcome through the development by Dr. Thomas Graf of an avian RNA tumor virus plaque assay which can be used both at low and high (permissive and non-permissive) temperatures. The choice of AMV for this research proposal is based upon several important considerations. (1) AMV is a C-type virus which reproducibly induces leukemia. (2) The host range of AMV is extremely restricted; only chicken, quail, and turkey cells are affected. This ensures the safety of laboratory personnel. (3) AMV and AMV-producing cells (myeloblasts) can be obtained in very high titers and quantities from the blood of infected chicks. (4) AMV-producing myeloblasts can be grown in large quantities in tissue culture for extremely long periods of time with continual yields of high titers of AMV. This combination of the properties of AMV and of the cells in which it replicates makes AMV an ideal C-type virus for the broad investigation which I am proposing. No other C-type virus shares all of these advantages so necessary for my approach to the problem.

9. Details of experimental design and procedures (append extra pages as necessary)

Please see the appended pages. The research proposal and supporting information are organized as follows:

1. AIM AND METHOD OF STUDY

Specific aims (a more detailed explanation than allowed under Section 7)

Plan of attack
- Infected chick myeloblasts producing AMV
- Isolation of AMV virions
- Radioactively labeled AMV virions
- Temperature sensitive and conditional lethal mutants of AMV
- Isolation of high molecular weight AMV RNA
- Eukaryotic *in vitro* amino acid incorporation systems
- Characterization of products synthesized *in vitro* under the direction of wild-type and mutant AMV RNA

The significance of the studies proposed

2. WORK ACCOMPLISHED BY ME
- Unpublished results
- Published results

WORK ACCOMPLISHED BY OTHERS
- Temperature sensitive and conditional lethal mutants of RNA-containing eukaryotic viruses
- AMV RNA as mRNA
- Virion-associated components and virus-specific proteins as possible AMV RNA coded products

References

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1. AIM AND METHOD OF STUDY

Specific aims: Using the recently developed plaque assay of Graf (1) for avian leukosis viruses, temperature sensitive and conditional lethal mutants of the oncogenic C-type avian myeloblastosis virus (AMV) will be sought. The mutants will be characterized by measuring the uptake in tissue culture of radioactive precursors into the proteins, glycoproteins, glycolipids, lipids, RNA, and DNA of AMV virions at both the permissive and nonpermissive temperatures. These techniques will allow the classification of AMV temperature sensitive and conditional lethal mutants into separate groups defective (at the nonpermissive temperature) in the biosynthesis of the several classes of macromolecules. Pairs of independently isolated mutants will be grown together at the nonpermissive temperature under various conditions in order to determine recombination and complementation groups. The high molecular weight RNA from wild-type AMV and from individual members of the various classes of mutant virions will be isolated and used as mRNAs in highly purified, initiation factor- and mRNA-dependent *in vitro* amino acid incorporation systems derived from several eukaryotes (2,3). The products synthesized at both the permissive and the nonpermissive temperatures under the direction of wild-type AMV RNA and of temperature sensitive and conditional lethal mutant AMV RNA will be compared by numerous techniques in order to identify and characterize the gene product corresponding to the mutation in the AMV genome. Analyses of the defects in macromolecular biosynthesis studied in tissue culture, the determination of recombination and complementation groups, and analyses of the products synthesized *in vitro* under the direction of wild-type and mutant AMV RNA should provide considerable knowledge of the mechanisms of virus-induced cell transformation, viral replication, and lead to a solution of the problem of which of the AMV RNA gene products is responsible for the induction, maintenance, and proliferation of the virus-induced neoplastic disease.

Plan of attack: Although temperature sensitive and conditional lethal of RNA-containing eukaryotic viruses have been exploited for only 3 or 4 years, they have proven to be extremely valuble tools for the investigation of virus-mediated cell transformation and of viral replication. No temperature sensitive or conditional lethal mutants of AMV have ever been isolated because of the lack of a suitable assay and screening system. The plaque assay developed recently by Graf (1) for avian leukosis viruses now makes the isolation of such mutants possible. Graf's assay is suitable for avian leukosis virues of subgroups B and D (1); AMV is known to consist predominantly of viruses of subgroup B (4). AMV has a very restricted host range, being confined to chickens, Japanese quail, and turkeys (5). This is one reason that I have chosen to work with this C-type virus; my laboratory does not have the complex safety facilities required for experimentation with viruses which can transform, and/or replicate in, mammalian cells. A second reason for the choice of AMV is that the virus accumulates in extremely high titers both in the plasma of sick chicks (6-8) and in tissue culture media (6,9). These properties, which other C-type viruses do not share, make the growth of large quantities of the virus and the purification of the viral RNA in mg quantities both safe and possible.

Infected chick myeloblasts producing AMV. Chicks (strain Shaver Starcross), younger than 24 hours post-hatching, will be inoculated interperitoneally with 5×10^9 AMV virions (BAI strain A, predominantly subgroup B) per

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chick in 0.5 ml of phosphate buffered saline; this insures the development of myeloblastosis in greater than 75% of the recipient chicks within 3 weeks (6). Blood smears from each chick will be inspected every few days starting 14 days after inoculation. When the myeloblast count in the blood reaches greater than 1.5×10^9 myeloblasts per ml of blood (equivalent to 25-40% of the blood volume), the chicks will be bled by cardiac puncture. The blood will be mixed immediately with heparin and chilled. After washing the myeloblasts by gentle centrifugation, the cells will be maintained in tissue culture (6,9).

Isolation of AMV virions. Bulk preparations of unlabeled wild-type AMV will be obtained from the plasma of chicks which show high titers of myeloblasts in blood smears 14-21 days following inoculation. Such plasma routinely contain greater than 5×10^{11} AMV virions per ml, and the isolation techniques for highly infectious AMV from such material are already standard operations in the laboratory (6). Mutant strains of AMV which have been selected for further study will be grown at 36° in tissue culture using bone marrow cells from healthy chickens and/or cells from chick embryos (see below). As in the case of AMV grown in tissue culture for the production of radioactively labeled AMV (see below), the tissue culture media will be harvested at 24 hour intervals and the AMV-producing cells returned to tissue culture with fresh media. This process will continue until sufficient material (approximately 10^{13} AMV virions) is accumulated to allow the isolation of about 0.1 mg of high molecular weight AMV RNA (6). The titer of AMV virions in tissue culture media, harvested every 24 hours, is in the range of $3\text{-}5 \times 10^9$ AMV per ml (6). AMV-producing cells may be kept in tissue culture for longer than 6 months while virus production continues undiminished (9). Mutant and radioactively labeled AMV virions will be isolated from tissue culture media pooled from repetitive harvests by techniques already developed in the laboratory (6). The virions will be stored under the conditions described by Smida and Smidova (10).

Radioactively labeled AMV virions. For the production of labeled virions, the tissue culture medium will contain appropriate radioactive precursors to protein, glycoprotein, glycolipid, lipid, RNA, and/or DNA. The medium, containing newly released AMV virions, will be separated from the myeloblasts or from the bone marrow cells from healthy chickens and/or cells from chick embryos by low speed centrifugation under sterile conditions every 24 hours. The AMV-containing medium will be saved, and the AMV-producing cells will be returned to tissue culture with fresh medium and radioactive label(s) for subsequent harvesting of radioactive AMV virions at periodic intervals. The media from repetitive harvests will be pooled and the AMV virions isolated as described above.

Temperature sensitive and conditional lethal mutants of AMV. For the induction of temperature sensitive and conditional lethal mutants of AMV, the AMV-producing infected chick myeloblast cultures will contain in the medium those mutagens (azacytidine, fluorouracil, nitrosoguanidine, etc.) which already have proven effective in generating temperature sensitive and conditional lethal mutants in other eukaryotic RNA-containing viruses. Isolated, purified, and concentrated AMV virions (see above) will be treated *in vitro* with proven and effective mutagenic agents (γ rays, ultraviolet light, nitrous acid, etc.). The dose of each mutagen or mutagenic agent will be regulated so as to give approximately a 5% survival of the AMV population. Untreated AMV controls and the mutagenized AMV stocks will be assayed by the use of the plaque assay technique of Graf (1). Individual plaques arising at 36° (chosen as the permissive temperature) from the mutagenized AMV stocks or from

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the mutagenized AMV-producing myeloblast cultures will be reassayed at 41° (chosen as the nonpermissive temperature). AMV will be recovered from individual plaques produced at 36° and then replaqued at 41°. If the titer of recoverable AMV virions from the original 36° plaque is too low to be assayed accurately at 41°, the cells and the AMV from the plaque will be subcultured at 36° in the presence of additional cells from healthy chicken bone marrow and/or from chick embryos. AMV virions will be recovered from these cultures, concentrated, and then plaque assayed at 41°. AMV from individual plaques produced at 36° which do not produce plaques at 41° will be selected and replaqued on multiple plates at 36° at high dilution. The material in the rare single plaques will be recovered and the AMV contained in the region of such plaques, theoretically representing a homogeneous population, will be grown as described under Isolation of AMV virions to yield mutant AMV stocks with a sufficient number of virions to enable high molecular weight AMV RNA preparations to be made (see below). Large numbers of each mutant will be plaqued at 41° in order to determine the reversion rate of each temperature sensitive and conditional lethal mutant. Only those mutant strains which exhibit genetic stability will be used for further studies.

The genetic defects in the AMV temperature sensitive and conditional lethal mutants will be characterized in tissue culture using bone marrow cells from healthy chickens and/or cells from chick embryos by measuring the uptake of radioactive precursors into proteins, glycoproteins, glycolipids, lipids, RNA and/or DNA of the AMV virions and into virus specific products at both the permissive and nonpermissive temperatures. Culture media will be separated periodically from the cells and the AMV virions in the media will be purified, concentrated, and assayed by counting plaques formed at 36° and 41°, by inoculation into newly hatched chicks (normal temperature = 41°), and by physical techniques such as equilibrium centrifugation in preformed sucrose density gradients. Material banding at the bouyant density of AMV (1.16-1.18 g per ml) will be analyzed by the presence of radioactivity in protein, and/or glycoprotein and/or glycolipid, and/or lipid and/or RNA and/or DNA, by the level of ATPase activity, and by quantitative electron microscopy in order to determine the extent of formation of non-infectious AMV virions. These techniques will allow the classification of the AMV temperature sensitive and conditional lethal mutants into separate groups defective (at the nonpermissive temperature) in the synthesis of the several classes of macromolecules. Pairs of independently isolated mutant strains of AMV will be inoculated into newly hatched chicks, assayed together at high multiplicities of infection by Graf's (1) plaque assay at 41°, and cultured together at high multiplicities of infection at 41° with bone marrow cells from healthy chickens and/or with chick embryo cells in order to determine recombination and recombination groups.

Isolation of high molecular weight AMV RNA. Isolated, purified, and concentrated AMV virions will be disrupted with dithiothreitol followed immediately by the addition of sodium dodecyl sulfate and phenol extraction at pH 9 to protect (11) the poly A-rich regions in the high molecular weight AMV RNA and to insure that the RNA is extracted efficiently into the aqueous phase (6,12). Chicken liver tRNA, isolated and highly purified in this laboratory, will be used as a carrier and protective agent during the isolation of the AMV RNA (6). The RNA in the aqueous phase following phenol extraction will be extracted with diethyl ether to remove residual phenol and then precipitated with 2 volumes of 95% ethanol at -20°.

1½% of the high molecular weight AMV RNA is known to contain several

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poly A-rich regions ranging in lengths between 100 and 200 nucleotides (13-15). Such poly A-rich regions exist in all eukaryotic mRNAs, and in all eukaryotic viral mRNAs including the RNA contained in eukaryotic C-type viruses; the only exceptions are the mRNAs for histones, which have uniquely short half-lives (16). The AMV RNA preparations, containing the added chicken liver tRNA, will be dissolved in 10 mM Tris-HCl (pH 7.6 at 20°), 0.2 mM $MgCl_2$, 0.5 M KCl and applied at 23° to columns of microcrystalline cellulose (17). These columns will be washed with the application buffer; the low molecular weight RNA components endogenous in AMV RNA preparations and the added chicken liver tRNA are not retained by the columns (6). Undegraded 70S AMV RNA will then be eluted from the columns by reducing the KCl concentration in the buffer from 0.5 M to 0.1 M (6). Zone velocity centrifugation of such AMV RNA preparations, either unlabeled or labeled, through preformed density gradients of ribonuclease-free sucrose indicates that this material represents undegraded 70S high molecular weight AMV RNA in pure form (6).

Eukaryotic *in vitro* amino acid incorporation systems. The control (wild-type) and the various mutant AMV RNA preparations will be used as mRNAs at both the permissive and nonpermissive temperatures in highly efficient *in vitro* amino acid incorporation systems developed in this laboratory (2,3,6). These systems are derived from eukaryotic sources such as rabbit liver, rabbit reticulocytes, chicken liver, chicken reticulocytes, AMV-producing chick myeloblasts, 11 to 14-day old chick embryo leg muscles, and rat liver. All of these systems can be made completely dependent upon several exogenous protein synthesis initiation factors (2,3,18-21), tRNA (2,3), and mRNA (2,3,6). Homologous preacylated tRNA is used in each system, and each system has been shown to translate properly both the α and the β rabbit globin mRNAs (2,3).

The availability of heterologous *in vitro* amino acid incorporation systems is of paramount importance since group-specific antigens corresponding to the avian leukosis-sarcoma complex are found in "leukosis-free" chick embryos (22-25); this property of chickens is inherited as a single Mendelian dominant gene (26), and bears a striking resemblance to a similar situation found in mice which can synthesize group-specific antigens while exhibiting no detectable complete replicating leukemia viruses (27). These phenomena are consistent with the "virogene" and "oncogene" concepts of Todaro and Huebner (28), as are the AMV RNA-host cell DNA annealing data of Baluda and coworkers (29) which point to the presence of AMV genes in the DNA of both phenotypically normal and AMV-infected chicken cells.

Characterization of products synthesized *in vitro* under the direction of wild-type and mutant AMV RNA. Radioactive products synthesized *in vitro* at both the permissive and nonpermissive temperatures under the direction of wild-type AMV RNA or of mutant AMV RNA will be mixed in independent experiments with components obtained from wild-type AMV virions and with components from the mutant AMV virions from which the mutant RNA was extracted. In most cases, the AMV virions will have been radioactively labeled in tissue culture; however, some AMV virion preparations will be labeled *in vitro*, either before or after disruption of the virions with Triton X-100, with ^{125}I using the lactoperoxidase iodination reaction which specifically labels histidine and tyrosine residues (30-33). The ribonucleoprotein cores from AMV virions will be isolated by the method of Stromberg (34) using Sterox SL; in most experiments the components of the cores will be labeled in tissue culture, but for some experiments the proteins of the cores will be labeled *in vitro* with ^{125}I as described above.

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Many of the techniques for the identification and/or purification and/or measurement of C-type viral group-specific and type-specific antigens and of enzymatic activities will be discussed under WORK ACCOMPLISHED BY OTHERS, subsection Virion-associated components as possible AMV RNA coded products. Additional methods which will be used will include affinity chromatography of viral glycoproteins (35) and direct radioimmunoassay of group-specific antigens (a technique which is 100-fold more sensitive than complement fixation and 1,000-fold more sensitive than gel immunodiffusion) (36). Group-specific antigens will also be detected by the double antibody method (37), by immunoelectrophoresis (38), by N-terminal amino acid sequence data (39,40), and by isoelectric focusing (41). Antisera containing antibodies directed against purified AMV components will be produced by standard techniques in hamsters, pigeons, rabbits, rats, or guinea pigs. Also to be used for the fractionation of AMV components will be the standard techniques of ion exchange chromatography upon modified celluloses and hydroxyapatite, polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, and gel filtration through Sephadex and through Sepharose in the presence and absence of denaturing agents such as urea, acetic and formic acid, sodium dodecyl sulfate, and guanidine hydrochloride. All AMV component fractionation experiments will be of the double-label variety; one radioactive label (^{3}H, ^{14}C, or ^{35}S) will be incorporated into the products synthesized in one of the *in vitro* amino acid incorporating systems under the direction of the RNA extracted from either the wild-type AMV or from one of the AMV temperature sensitive or conditional lethal mutants and the second radioactive label (^{3}H, ^{14}C, ^{35}S, or ^{125}I) will be contributed by viral components from either wild-type AMV virions or from temperature sensitive or conditional lethal mutant virions of AMV grown in tissue culture or by wild-type or mutant viral components labeled *in vitro*. Correspondence between the 2 radioactive labels in protein will be confirmed by subjecting the material to digestion with trypsin, chymotrypsin, subtilisin, papain, pepsin, or pronase, followed by analysis of the resultant peptides by 2 dimensional paper electrophoresis and chromatography (42) and autoradiography, and by gradient elution chromatography upon columns of Dowex 50 (43).

The significance of the studies proposed: About 5 years ago, a line of cells (RD cells), which neither contained nor produced C-type particles, was established in tissue culture (44). These cells, originally obtained from a human rhabdomyosarcoma, were inoculated prenatally into 4 kittens and subsequently produced disseminated rhabdomyosarcomas consisting of cells with a human karyotype (45). 2 of the cat tumors (1 of which was a brain tumor) and a tissue culture line (RD-114; a mixture of 2 morphologically distinguishable cell types) established from the brain tumor did, however, produce a C-type virus (RD-114). Several lines of evidence indicate that the RD-114 virus may be of human origin. The cells from which the virus buds have a human, rather than a feline, karyotype; the viral components cross-react with the #3 group-specific (interspecies-specific) antigens of C-type viruses from numerous sources, but the #1 group-specific (species-specific) antigen does not react in complement fixation tests with antisera to the #1 group-specific antigens of feline, murine, hamster, rat, avian (46), or Russell's viper (47) C-type viruses. It is of interest that the #1 and the #3 group-specific antigens are associated with the same protein located in the internal ribonucleoprotein core of the virus (48). RD-114 does not interfere with the transforming properties of feline

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C-type viruses of subgroups A, B, or C; nor is it inactivated by antisera to feline C-type viruses (46). The RD-114 virus has the typical physical properties of a C-type particle: bouyant density in sucrose = 1.16-1.18 g per ml, contains 70S RNA and a RNA-dependent DNA polymerase, and is about 100 nm in diameter with C-type morphology (46). RD-114 does not transform any cell line tested (including human), but it will replicate in human and canine cells (46). The RNA-dependent DNA polymerase is distinguishable immunologically from the corresponding enzyme from C-type viruses from all sources including lower mammals and primates (49,50). More recent information concerning the origin of RD-114 indicates that cats may possess 2 distinct types of C-type viruses, and that RD-114 may belong to the newly discovered second type (51). McAllister et al. (51) have induced the formation of a C-type virus (CCC virus) from a continuous feline cell line (CCC) in the presence of iododeoxyuridine. The group-specific antigenic properties of this new virus are related to the corresponding antigen of RD-114. The new virus is able to replicate in RD cells and other human or monkey cells, but is unable to grow in feline cells (51). This new evidence lessens the probability that RD-114 is an etiological agent in human cancer.

Strong evidence that C-type viruses are associated with human breast cancer has been accumulated in recent years. The electron microscope has been used to detect C-type particles in human milk (52-55), in thin sections of breast tumor tissue and in pleural effusion cells from breast cancer patients (56). These particles possess the distinguishing features of C-type viruses (57-61), although they also are found in milk from apparently normal women (62) and epidemiological studies have failed to establish an etiological role for these virus particles in human breast cancer (63). On the other hand, C-type virions from human milk have been produced continuously for periods of time longer than a year by co-cultivation with human embryonic cells in tissue culture (64). It also is of significance that 70S RNA-containing C-type particles from extracts of malignant human breast adenocarcinomas (but not of normal breast or of benign breast fibroadenomas) can use their virion-associated RNA-dependent DNA polymerase to synthesize DNA which contains nucleotide base sequences which are complementary to the RNA from mouse mammary tumor virus (65). RNA obtained from human breast adenocarcinomas (but not RNA from normal breast tissue or from such benign pathologies as fibrocystic disease or fibroadenoma) also possesses base sequences homologous to the base sequences in mouse mammary tumor virus RNA (66), but not to the base sequences in Rauscher leukemia virus RNA (65). None of the C-type particles associated with human breast cancer has been shown to be infectious or transforming for human cells.

RNAs extracted from human leukemia cells, sarcomas, and lymphomas have been shown to have base sequences homologous to the RNA from Rauscher leukemia virus (67-69). C-type particles containing 70S RNA and an endogenous RNA-dependent DNA polymerase have been obtained from the peripheral white blood cells of patients suffering from acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia. These C-type particles from human neoplastic cells or tissues are able to synthesize DNA with base sequences complementary to the RNA from Rauscher leukemia virus, but not to the RNA from mouse mammary tumor virus or to the RNA from AMV (70).

To summarize these results, the RNA in C-type viruses from human breast cancers is at least partially homologous with the RNA from a C-type virus known to induce mammary cancer in mice (mouse mammary tumor virus), but not

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to RNA from a known C-type leukemia virus (Rauscher leukemia virus). In contrast, the RNA in C-type viruses from human leukemia cells is at least partially homologous with the RNA from a C-type virus known to induce leukemia in mice (Rauscher leukemia virus), but not to RNA from a C-type virus known to cause mammary tumors in mice (mouse mammary tumor virus) or to the RNA from a C-type virus known to induce myeloblastic leukemia in chickens (AMV).

The analyses of AMV-specific macromolecular biosynthesis and the determination of recombination and complementation groups through the use of temperature sensitive and conditional lethal mutants of AMV, coupled with an identification and characterization of virion-associated and virus-specific products synthesized in vitro under the direction of the wild-type and of the mutant AMV high molecular weight RNA genomes, will allow many insights into the mechanisms of C-type virus-induced cell transformation and viral replication. These approaches appear appropriate for an identification and characterization of the AMV gene product(s) which lead(s) to the induction, maintenance, and proliferation of the myeloblastic leukemia. Because the biochemistry and molecular biology of higher eukaryotic life forms are so similar, such results should have relevance to the control of neoplastic growths in man.

2. WORK ACCOMPLISHED BY ME

Unpublished results. A strain of AMV (BAI strain A, predominantly subgroup B) and a strain of White Leghorn chickens (Shaver Starcross) have been found which together yield a very high incidence of myeloblastosis in young chicks (6). Techniques have been developed for the isolation of the AMV virion both from plasma and from tissue culture media, thus allowing both the purification of unlabeled virus in very large quantities and radioactively labeled labeled virus in reasonable and workable quantities (6).

Extremely effective techniques have been modified from published methods for the isolation of the high molecular weight RNA genome of AMV. The adsorption to, and desorption from, microcrystalline cellulose of the AMV 70S RNA, dependent upon the presence of poly A-rich regions in the high molecular weight RNA, has proven to be particularly useful (6). Personnel in my laboratory also have used this method successfully for the purification and isolation of undegraded mRNAs for rabbit and chicken globins and for chick embryo myosin (3,6,71). Using poly A of known chain lengths (72), the chain length dependence of poly A retention by microcrystalline cellulose under standard conditions and the ionic strength dependence of such retention by poly A chains of known chain lengths were determined (6). At 23°, in 10 mM Tris-HCl (pH 7.6 at 20°), 0.2 mM $MgCl_2$, 0.5 M KCl, poly A chains of 50 or more residues are retained by microcrystalline cellulose. Chains of poly A containing more than 50 residues could be fractionated according to length by gradually reducing the ionic strength. Experiments with pure lignin have shown that the suggestion of Delarco and Guroff (73) that the adsorption of purine-rich regions of RNA to cellulose is due to a "lignin-like" contaminant is without merit (6).

Preliminary experiments, based upon the data relating ionic strength and the chain length of poly A retained by microcrystalline cellulose, have indicated that mRNA from chick embryo leg muscle polysomes (which contains the mRNA for myosin) can be clearly separated on columns of microcrystalline cellulose by a reverse salt gradient, and that the 4S and the 4-6 30-40S RNAs resulting from the denaturation of the 70S AMV RNA can be fractionated by essentially the same technique (6). This is an extremely important development,

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since even high resolution electrophoresis through polyacrylamide gels and chromatography upon modified celluloses or methylated albumin-coated kieselguhr have not been successful in this type of fractionation.

Chicken and rabbit globin mRNAs, chick myosin mRNA, and 70S AMV RNA have been used as mRNA in the in vitro eukaryotic amino acid incorporation systems available in my laboratory (2,3,6) with excellent results. Several variables which usually are not controlled well in in vitro systems have been found to be extremely important for the translation of natural and viral mRNAs, exclusive of the effects of mRNA-specific initiation factors. We have confirmed the observation of Olsnes et al. (74) that low levels of non-ionic detergents increase the efficiency of in vitro translation. Olsnes et al. used Triton X-100 and Brij-58 in a rabbit reticulocyte in vitro system which is practically devoid of membranous material; we have found that Nonidet P-40 is even more effective (6). The ionic concentration and composition of both mono- and divalent cations have been found to be critical during in vitro translation. For example, under certain conditions, only the mRNA for the rabbit β globin chain is translated, while under other conditions, the translation of the mRNA for the rabbit α globin chain is favored (3).

My laboratory has continued work on the identification, purification, and characterization of eukaryotic protein synthesis initiation factors (18-21). All of the data published from my laboratory has since been confirmed by workers in other laboratories (75-80). The results essentially are not in agreement, with the possible exception of "M_3", with the very numerous publications from the group working with Dr. W. F. Anderson beginning in late 1969 and continuing to the present.

We have several manuscripts in preparation which describe further the properties, functions, and improved purifications of 2 eukaryotic initiation factors (IF-I and IF-II) (71). IF-I is a large protein (molecular weight = 300,000), obtained from high salt washes of polysomes, which can be reversibly inactivated by sulfhydryl reagents. The first function of IF-I, which has been purified almost to homogeneity (about 500-fold relative to the polysomal wash), is to bind specifically the eukaryotic initiator tRNA (Met-$tRNA_i^{Met}$) in a GTP-dependent reaction; the formation of this ternary complex is AUG- and magnesium ion-independent, activated by thiols, and is exceptionally stable at physiological temperatures; its formation is blocked by very low concentrations of the initiation inhibitor aurintricarboxylate. The second function of IF-I is to bind the initiator tRNA, in an AUG- and mRNA-independent reaction, to the 40S ribosomal subunit. There is no tissue or species specificity in these reactions with respect to either the initiator tRNA or IF-I (tissues tested: rabbit liver, rabbit reticulocytes, chicken liver, chicken reticulocytes, chick embryo leg muscles, and rat liver) (21). The N-formylated derivative of the initiator tRNA, which is not a natural initiator tRNA in the cytoplasm of eukaryotes, binds to IF-I to only 1/10 the extent of the natural initiator tRNA (21). In the presence of IF-II, which also is a large protein (molecular weight = 400,000) sensitive to sulfhydryl reagents, and separable from IF-I by ion exchange chromatography on DEAE-cellulose, the initiator tRNA-40S ribosomal subunit complex is joined by a 60S ribosomal subunit to form an 80S initiation complex (81). This reaction is stimulated by the AUG codon, but does not require natural or viral mRNAs (81). We have developed 2 in vitro amino acid incorporation systems, both dependent upon exogenous mRNA, from rabbit reticulocytes by 2 different techniques. One system cannot translate the mRNA for rabbit α globin although large quantities of rabbit β globin are synthesized; the second system synthesizes both globin chains efficiently (3). We are now

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screening the second system for the α globin mRNA-specific IF-III initiation factor in order to assay it in the first in vitro system. This mRNA-specific initiation factor will be purified and its precise role in controlling the translation of a particular mRNA investigated. This will be the first demonstration of an mRNA-specific IF-III which absolutely regulates the translation of a specific mRNA.

We also are concentrating on developing an in vitro amino acid incorporation system, as efficient as the other eukaryotic systems used in the laboratory, from AMV-producing chick myeloblasts (6). We are isolating, purifying, and characterizing protein synthesis initiation factors from chick embryos, other chicken tissues, and AMV-producing chick myeloblasts. The complete translation of the AMV genome may well depend upon the availability of specific initiation factors which regulate the efficiency and temporal expression of the AMV RNA. When available, these specific initiation factors from chickens will be used in a heterologous in vitro amino acid incorporation system because of the possibility of endogenous synthesis of group-specific antigens and perhaps other products specified by C-type viral information inherited by the chick embryo, the chick, and the adult chicken.

Published results: Published material, articles in press, and a manuscript submitted for publication are listed below and copies or reprints are enclosed with this proposal.

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2. The Isolation and Physical Properties of the Ribosomal Ribonucleic Acid of Escherichia coli, Stanley, W. M., Jr. and Bock, R. M., Biochem. 4 1302 (1965).
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WORK ACCOMPLISHED BY OTHERS

Temperature sensitive and conditional lethal mutants of RNA-containing eukaryotic viruses. Temperature sensitive and conditional lethal mutants have been successfully isolated from stocks of Rous sarcoma virus (82-89), Sindbis virus (90), vesicular stomatitus virus (91), reovirus (92), and influenza virus (93). Studies upon some of the temperature sensitive mutants of Rous sarcoma virus have shown that the transformation of the infected cell is reversible, and that the maintenance of the transformed state of the cell requires the constant expression of a viral gene which is not required for virus multiplication (84-89,94). Other studies have demonstrated that the transformed cell morphology expressed at low temperatures (36°) is associated with a drop in the intracellular concentration of cyclic AMP (95). Lai and Duesberg (96) have investigated the glycoproteins and glycopeptides from temperature sensitive mutants of Rous sarcoma virus and find that the glycoproteins from the virion grown at the permissive temperature (36°) have lower electrophoretic mobilities than those from the virion grown at the nonpermissive temperature (41°). It also was reported that the glycopeptides derived from virions released from transformed cells possess higher molecular weights than those from virions released from phenotypically normal cells (96). Some temperature sensitive mutants of Rous sarcoma virus express their defects by altering cell morphology (82), while some conditional lethal mutants cannot replicate at 41°; these mutants may express their defects either early (ts 149) or late (ts 75) in the infectious process (97).

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Using conditional lethal mutants of vesicular stomatitus virus, Szilagi and Pringle (91) demonstrated that in the group I mutants the mutated gene is the structural gene for the virion-associated RNA-dependent RNA polymerase.

Analyses of conditional lethal mutants of reovirus have revealed 3 recombination and coincident complementation groups (C, D, and E) (98). All mutants in the 3 groups are defective in the synthesis of double-stranded RNA at the nonpermissive temperature (39°), although the infected cells continue single-stranded mRNA synthesis (98). Mutants falling in groups C and D abruptly shut off double-stranded RNA synthesis upon being shifted from 31° to 39°; mutants in group E continue double-stranded RNA synthesis at the normal rate following such a temperature shift (98). After a physical analysis of the RNA species synthesized at the 2 temperatures by the mutants, a model was proposed to explain the nature and consequences of the mutations (99). It was concluded that group D mutants are defective in the structural gene(s) for virion polypeptide chain(s) (100). Other workers have investigated conditional lethal mutations in 5 cistrons of reovirus (101). Different mutants produce different amounts of viral polypeptides, but all of the mutants are able to synthesize all of the major viral polypeptides as judged by molecular weights and immune precipitations, although these polypeptides cannot be assembled into a virion structure (101). Reovirus mutants also have been studied which are defective in the ability to cleave precursor viral polypeptides into the final virus-specific proteins (102). Mutants of Sindbis virus (103) and of polio virus (104) also have been isolated and found to be defective in the processing of precursor high molecular weight viral polypeptides.

No temperature sensitive or conditional lethal mutants of AMV have ever been isolated.

AMV RNA as mRNA. There is remarkably little information on the use of AMV RNA as mRNA in *in vitro* amino acid incorporation systems. The information summarized below, and the fact that high molecular weight AMV RNA, not phenol extracted and containing about 6% protein, has been reported to be infectious in young chicks (105) (a report that has not been extended or confirmed either by the original workers or by workers in other laboratories), strongly supports the idea that the RNA contained in the AMV virion is the *plus* strand; i. e., able to be translated correctly by polysomes to yield virus-specific products. AMV RNA has been used as mRNA in *in vitro* amino acid incorporation systems derived from *Escherichia coli*, a prokaryote (106-110). In the first 4 publications, no attempt was made to identify or to characterize the products made *in vitro*. These studies are therefore suspect, since it has been shown that the addition of high molecular weight RNA to an *in vitro* system can stimulate the synthesis of endogenous products without the exogenous RNA itself being translated (111). Siegert *et al.* (110) have made the only analyses of the products synthesized under the direction of AMV RNA in *E. coli* extracts; they were able to identify the #4 group-specific antigen immunologically and to make a preliminary identification of the #1, #2, and #3 group-specific antigens solely on the basis of molecular weights.

Gielkens *et al.* (112) have used Rauscher leukemia virus RNA and mouse mammary tumor virus RNA as mRNAs in an *in vitro* system from *E. coli*. The products made *in vitro* were co-electrophoresed with the respective virion proteins, but the results were not particularly convincing that virus-specific proteins had in fact been synthesized. Gielkens *et al.* (112) also mentioned that attempts to translate these viral RNAs in oocytes or in a rabbit reticulocyte *in vitro* system were unsuccessful. In contrast to these negative results, Vidrine *et al.* (113) have reported that Rauscher leukemia virus

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RNA is an effective mRNA in extracts of Rauscher leukemia virus-infected tissue cultured cells originally obtained from mice. However, no evidence for the synthesis of virus-specific products was presented. The same group of workers subsequently demonstrated that Rauscher leukemia virus RNA would enter into polysomes from Rauscher leukemia virus-infected cells in the presence of a high salt wash of homologous polysomes (114). Such washes of polysomes are known to contain, amoung other ribosomal proteins, initiation factors for protein biosynthesis.

There is no published information concerning the translation of AMV RNA in in vitro amino acid incorporation systems derived from eukaryotes.

Virion-associated components and virus-specific proteins as possible AMV RNA coded products. The proteins (115-117), glycoproteins (118-121), and, to a lesser extent, the glycolipids (120) and phospholipids (122) of the viruses comprising the avian leukosis-sarcoma complex have been fairly well characterized. Most attention has been focused on the type-specific antigens (located in or on the external envelope of the virion) (118-121, 123-132) and on the group-specific antigens (located in the internal ribonucleoprotein core) (119, 133-140). The nucleoprotein core itself has been isolated and extensively studied (34, 141-145).

There are, however, components associated with the AMV virion which are not as well known or as well characterized. The following entities and/or activities have been reported to be associated with the AMV virion: ATPase (146), RNAase (147-150), DNA-specific endonuclease (151,152), RNA methylase (153), RNA-dependent DNA polymerase and RNAase H in a single subunit (152,154-156), a protein that stimulates the RNA-dependent DNA polymerase (157), aminoacyl-tRNA synthetases (158), specific ribosomes (159) with their ribosomal RNA and approximately 63 individual proteins (160), 7S DNA (161,162), about 20% of the RNA content of AMV which represents 4S RNA which does not accept amino acids and 4S tRNA which accepts amino acids, is functional in protein synthesis and is derived, but distinct from, the host cell tRNA (163-172), and 4S tRNA-like RNA which is released from the high molecular weight AMV 70S RNA genome only under denaturing conditions (173).

In addition to most of the above components, the Rous sarcoma virion has been reported to be associated with DNA ligase (174), DNA-specific exonuclease (174), nucleotides (175), nucleotide kinase (175), phosphatase (175), hexokinase (175), and lactate dehydrogenase (175).

Finally, an RNA-dependent RNA polymerase, which prefers AMV RNA as a template, has been reported to exist in AMV-infected myeloblasts (176,177). However, there is disagreement concerning the intracellular presence of RNA complementary to the RNA in the AMV virion. Such complementary RNA has been reported to be located in the nucleus of infected myeloblasts (178), although Baluda and Nayak have not been able to detect any cellular RNA complementary to the virion RNA (179,180).

In view of the large number of virion-associated components and the possible existence of an intracellular enzyme in infected myeloblasts involved in the replication of AMV RNA through a complementary RNA template, it is important to determine which of these entities are coded for by the RNA genome of AMV.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space: Two-room laboratory (rooms 468 and 470A in the Department of Molecular Biology, School of Biological Sciences, Steinhaus Hall, University of California, Irvine, California 92664); total of 1074 square feet.

Community space: Cold rooms (-20° and +4°), warm rooms (25° and 37°), a dark room and autoradiography and photographic supplies, a high voltage electrophoresis explosion-proof room, an animal facility (UCI Vivarium), a room for chickens, and the UCI Media Kitchen with autoclaves.

Equipment: L3-50 Beckman Spinco ultracentrifuge, ultraviolet and visible spectrophotometers (Beckman DB, Perkin-Elmer Coleman Hitachi model 124, Gilford models 240 and 2000), RikaDenki single pen recorder, Mettler H-10 semi-micro balance, Buchler refrigerated fraction collector, 2 Isco model 272 fraction collectors, Forma model 2095-2 heating and cooling circulating water bath, 3 Napco model 220 water baths, Barnstead model EPR-½C redistiller, metering pumps (Buchler Polystaltic, Sigmamotor peristaltic, Stalprodukter peristaltic, Beckman piston metering pump), Thermovac model FD-1-V2DG lyophilizer, Sears and Roebuck egg incubator and chick brooder, ultraviolet-irradiated sterile box, refrigerators (Coldspot small and regular size and Glenco large stainless steel), -20° freezer, Kelvinator series 100 -76° freezer, Buchler flash evaporator with vacuum pump, Thelco model 29 vacuum oven, National 37° forced air incubator, gel electrophoresis supplies (includes de-stainer and gel scanner), and thin layer and column chromatography supplies.

Community equipment: Scotsman chipped ice machine, Gilson Electrophorator model D high voltage electrophoresis equipment, Beckman amino acid analyzer, electron microscope, 1 L2 and 2 L2-65B Beckman Spinco ultracentrifuges, polyacrylamide gel slicer, American Optical Spencer model 1036 microscope, and autoclaves.

11. Additional facilities required:

The facilities or equipment required to carry out the research proposed are requested and justified in the budget under Section 14. My most pressing needs are for a scintillation spectrometer, for increased centrifugation capacity, and the equipment to allow bulk growth of cells and AMV in tissue culture and to screen and assay for mutants of AMV at the permissive and nonpermissive temperatures (i. e., 2 water-jacketed CO_2 incubators). My preliminary experiments have been carried out with make-shift and inadequate facilities. As may be seen from the list of equipment that I already possess, the laboratory is set up well for biochemical and molecular biology studies, but not for large-scale virus production or for careful and extensive tissue culture studies.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical sketch of principal investigator:

Name: Wendell M. Stanley, Jr.
Title: Associate professor, step II. 2/3 appointment in the Department of Molecular Biology and Biochemistry, and 1/3 appointment in the Department of Biological Chemistry, California College of Medicine, both on the Irvine campus.
Birthdate:
Place of birth: REDACTED
Nationality:
Sex: Male
Marital status:

REDACTED

Education:	Univ. of Cal., Berkeley	A.B.	1957	Chemistry & Biochem.
	Univ. of Wis., Madison	M.S.	1959	Biochemistry
	Univ. of Wis., Madison	Ph.D.	1963	Biochemistry
	N. Y. U. Sch. Med., N. Y.	U.S.P.H. postdoctoral fellow	1963-1965	Biochemistry

Employment: Instructor in Biochemistry, N. Y. U. Sch. Med., Dept. of Biochemistry, 1965-1966.
Assistant professor of Biochistry, N. Y. U. Sch. Med., Dept. of Biochemistry, 1966-1967.
Assistant professor of Biochemistry, Univ. of Cal., Irvine, Dept. of Molecular Biology and Biochemistry (2/3), and Dept. of Biological Chemistry, California College of Medicine (1/3), 1967-1970
Associate professor of Biochemistry, same as above, 1970-present.

Honors: Honors in Chemistry, Univ. of Cal., Berkeley, 1957.
Phi Beta Kappa, Univ. of Cal., Berkeley, 1957

Major research interest: Avian myeloblastosis virus, control of protein synthesis.

Role in proposed project: Principal investigator.

13. Publications (five most recent. Not included below are four manuscripts currently accepted for publication which are in press. A list of these may be found under WORK ACCOMPLISHED BY ME, Published results. A fifth manuscript will be communicated by Dr. Howard K. Schachman to the Proceedings of the National Academy of Sciences U. S. within the next week: Two Eukaryotic Protein Synthesis Initiation Factors (IF-I and IF-II) are Required to Form an Initiation Complex with Rabbit Reticulocyte Ribosomes, Cashion, L. M., Neal, P. M. and Stanley, W. M., Jr.)

1. An Economical Procedure for the Preparation of L-[^{35}S]Methionine of High Specific Activity, Graham, A. R. and Stanley, W. M., Jr., Anal. Biochem. 47 505 (1972).
2. Initiation of Protein Synthesis: The Interaction of Methionine-Specific Transfer Ribonucleic Acids with Rabbit Reticulocyte Ribosomes, Cashion, L. M., Kolb, A. J. and Stanley, W. M., Jr., Biochim. Biophys. Acta 262 525 (1972).
3. Recognition of Eukaryotic Initiator tRNA by an Initiation Factor and the Transfer of the Methionine Moeity into Peptide Linkage, Dettman, G. L. and Stanley, W. M., Jr., Biochim. Biophys. Acta 287 124 (1972).

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Name: Wendell M. Stanley, Jr.

Title: Associate Professor, Step [illegible]

4. Preparation and Analysis of L-[^{35}S]Methionine Labeled Transfer Ribonucleic Acids from Rabbit Liver, Stanley, W. M., Jr., Anal. Biochem. 48 202 (1972).
5. The Ternary Complex of Initiation Factor IF-I, Met-tRNA$_i^{Met}$ and GTP. An Aurintricarboxylate-Sensitive Intermediate in the Initiation of Eukaryotic Protein Syntheis, Dettman, G. L. and Stanley, W. M., Jr., Biochim. Biophys. Acta 299 142 (1973).

Sex: Male

[illegible]

Univ. [illegible]

[illegible]

[illegible]

[illegible]

13. Publications (five most recent). Not included below are four manuscripts currently [illegible]

[illegible]

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14. First year budget

A. Salaries (give names or state "to be recruited")	% time	† Amount
Professional (give % time of investigator(s) even if no salary requested)		
Wendell M. Stanley, Jr. (Principal Investigator)		
(3/9 summer salary, 3 months)		
Linda M. Cashion (PhD expected Sept. 1973)		
Glenn E. Brown (PhD expected Sept. 1973)		
Alfred J. Kolb (graduate student)		REDACTED
Bryn Henderson (graduate student)		
Peter Neal (graduate student)		
Technical		
Marcia E. Witte (Lab. Asst. I, Step III)*		
*Reflects 5% merit + 6% cost of living		
Kent Cooper (Laboratory Helper)		
Larry Dickinson (Laboratory Helper)		REDACTED
Steve Noble (Laboratory Helper)		
Retirement and other security benefits (total)		1,049
†All salary and benefit rates are set by University policies.	Sub-Total for A	16,279
B. Consumable supplies (by major categories)		
Replacement drive for Spinco L3-50 ultracentrifuge		500
Tissue culture media and supplies		3,000
Chemicals and biochemicals		2,000
Radioactive materials		3,500
Animals and animal boarding in UCI Vivarium		6,000
Glass- and plasticware		1,000
Radioactive counting supplies		1,000
Radioactive waste disposal		250
+ 6% Calif. sales tax (not on animal boarding)		795
	Sub-Total for B	18,045
C. Other expenses (itemize)		
Service contract for Spinco L3-50 ultracentrifuge		300
Artwork, photography, page costs, reprints		1,000
Repairs and maintenance on laboratory equipment		1,250
	Sub-Total for C	2,550
	Running Total of A + B + C	36,874
D. Permanent equipment (itemize)		
Beckman LS-330 scintillation spectrometer		16,000
Leeds and Northrup 7415-E2 research pH meter		650
Amicon DC2 hollow fiber dialyzer/concentrator		850
CRC Labwasher + installation		1,780
New Brunswick T-41 bottle roller for tissue culture		2,400
Two New Brunswick CO-20 CO_2 incubators		2,600
Beckman Spinco type SW-40-Ti rotor		2,650
+ 6% Calif. sales tax		1,616
	Sub-Total for D	28,546
E. Indirect costs (15% of A+B+C)	E	5,531
	Total request	70,951

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15. Estimated future requirements: Please see appended detailed future budget and justifications.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	17,724	20,324	3,100	4,441	6,172	51,761
Year 3	18,486	23,709	3,650	8,295	6,877	61,017

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R: REDACTED MATERIAL

Budget:

	Year 1	Year 2	Year 3
Personnel† (For consultants, see page 4f)			
W. M. Stanley, Jr. (Principal investigator) R	REDACTED		
REDACTED			
Marcia E. Witte (Lab. Asst. I, Step III) R	REDACTED		
REDACTED			
Laboratory Helpers (2) (50%)	REDACTED		
Retirement or other security benefits (total)			
Category total	REDACTED		
Permanent equipment			
Beckman LS-330 scintillation spectrometer	16,000	0	0
Leeds and Northrup 7415-E2 research pH meter	650	0	0
Amicon DC2 hollow fiber dialyzer/concentrator	850	0	0
CRC Labwasher + installation	1,780	0	0
New Brunswick T-41 bottle roller for tissue culture	2,400	0	0
Two New Brunswick CO-20 CO_2 incubators	2,600	0	0
Spinco type SW-40-Ti rotor	2,650	0	0
Sorvall RC2-B centrifuge with SS-34, GSA, and GS-3 rotors	0	4,190	0
Van Waters & Rogers vacuum pump	0	0	800
Amicon MMC concentrator	0	0	1,550
Barnstead PM-70CB conductivity meter with cell	0	0	525
Spinco type 75-Ti rotor	0	0	1,700
New Brunswick G76 Gyratory shaker and 3 platforms	0	0	1,000
Kelvinator -96° UC-540 chest freezer	0	0	2,250
+ 6% Califonia sales tax	1,616	251	470
Category total	28,546	4,441	8,295
Consumable supplies			
Replacement drive for Spinco L3-50	500	0	500
Tissue culture media and supplies	3,000	3,500	4,000
Chemicals and biochemicals	2,000	2,300	2,700
Radioactive materials	3,500	3,750	4,000
Animals and animal boarding in U. C. I. Vivarium	6,000	7,000	8,000
Glass- and plasticware	1,000	1,250	1,500
Radioactive counting supplies	1,000	1,250	1,500
Radioactive waste disposal	250	350	450
+ 6% California sales tax (not for animal boarding)	795	924	1,059
Category total	18,045	20,324	23,709
Miscellaneous			
Service contract for Spinco L3-50	300	350	400
Artwork, photography, page costs, reprints	1,000	1,250	1,500
Repairs and maintenance on laboratory equipment	1,250	1,500	1,750
Category total	2,550	3,100	3,650
Direct cost total	65,420	45,589	54,140
Three year direct cost total	.....		$165,149

†Number of personnel working on this project: 10. W. M. Stanley, Jr., Witte, M. E., Brown, G. E., Kolb, A. J., Cashion, L. M., Cooper, K. D., Henderson, B., Neal, P., Dickinson, L. and Noble, S.

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Justification for personnel:

Wendell M. Stanley, Jr. (Principal Investigator): Salary for the 3 summer months is requested in order that the continuity of the project be assured. No summer vacations will be taken. Also working through the summer months will be a full time technician, 3 (and perhaps 4) graduate students, and undergraduate employees (see below).

Marcia Witte (Full time laboratory assistant, presntly at step III): Ms. Witte holds a B.S. in Biology from the School of Biological Sciences, Univ. of Cal., Irvine. She has been a technician in my laboratory for 3 years and is fully trained in the operations in the laboratory. She is paricularly essential for the care of fertile eggs and chicks, bleeding chicks by cardiac puncture, working up virus preparations, preparing ribosomes, ribosomal subunits, and cell extracts, preparing samples for radioactive counting, and in the preparation of reagents and solutions. Her continued employment is considered essential for the smooth progress of this project; another technician hired at a later date would take a considerable amount of my time in explaining the nature of the project and in training him/her for his/her duties. It also would be difficult to find a replacement technician with the biological education of Ms. Witte.

Laboratory Helpers: Three part time student laboratory helpers are requested to perform duties which do not require the specialized training of a laboratory technician. Such duties include gathering and transporting dirty glass- and plasticware, putting clean glass- and plasticware back where is belongs, maintaining the laboratory in a satisfactory state of cleanliness, cleaning the chicken brooders and egg incubator, and feeding and watering the chicks and chickens. Laboratory helpers also will assist other laboratory personnel whenever such help is consisent with the abilities of the student helpers.

Justification for permanent equipment:

The California Division of the American Cancer Society ($10,000, "Control of Protein Biosynthesis in Mammalian Cells", 7/1/68-6/30/69), the Cancer Research Coordinating Committee of the Univ. of California ($5,000, "Control of Protein Synthesis in Normal and Tumorous Cells", 7/1/68-6/30/69), and the American Cancer Society ($84,583, "Control of Protein Biosynthesis in Mammalian Cells", 3/1/69-6/30/71) originally provided the funds to equip my new laboratory when I moved to the Irvine campus of the Univ. of California. Although the original research project partially overlaps the project which I am presently proposing, there are sufficient differences to require new and additional equipment.

Beckman LS-330 scintillation spectrometer: Many of the experiments proposed in this application will be of the double radioactive label variety (see the subsection entitled "Characterization of products synthesized in vitro under the direction of wild-type and mutant AMV RNA"). The use of a scintillation spectrometer therefore is a necessity. There are 2 single channel scintillation counters in the department; they are in the laboratories of newly recruited staff members. These counters were purchased when the Irvine campus opened about 7 years ago and they are used

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for teaching purposes. In 1967, classes were taught once each year and the averge class enrollment was about 35; this year corresponding classes are taught 3 times a year and the enrollment per class has soared to between 150 and 200. The departmental counters are old, often out of service awaiting repair, heavily used by class students, and cannot be depended upon for research purposes. The Beckmodel LS-330 is a very practical machine, stripped of non-essential gadgets such as digital presentation (replaced with a much cheaper ratemeter) and automatic quench correction. The LS-330 has 3 channels and quench corrections can be made either by external standard or by channels ratio. Data output is by teletype (serviced by Beckman, and not by the very troublesome teletype field engineers) and punched paper tape which is compatible with the computor services available at Irvine for the analysis of double label experiments.

Leeds and Northrup 7415-E2 research pH meter: An expandable scale pH meter of high sensitivity is required for the accurate determination of pH of tissue culture media and of other solutions. This model is cheaper than digital display instruments but does not sacrifice reproducibility or accuracy. The E2 designation indicates that the instrument will be equipped with a miniature combination electrode for the pH measurement of small samples. The 7415 also is combatible with specific ion electrodes which may be purchased later, especially for the measurement of divalent cation concentrations which are so important in in vitro amino acid incorporation systems.

Amico DC2 hollow fiber dialyzer/concentrator: This instrument will be used to remove ammonium sulfate after precipitation of AMV, to remove sucrose and to concentrate AMV following equilibrium centrifugation of the virus in preformed sucrose density gradients, and to concentrate suspensions of the purified virus. Many of the proteins to be purified are very labile in dilute solution. Normal dialysis leads to denaturation and extensive losses of activity and, in addition, must be preceded or followed by concentration steps which in themselves often result in denaturation and losses of activity. The DC2 also will be used to dialyze and concentrate fractions obtained from ion exchange chromatography, gel filtration, isoelectric focusing, and preparative polyacrylamide gel electrophoretic purification of viral components and of protein synthesis initiation factors. The instrument will be used to concentrate and adjust the ionic composition and concentration of eukaryotic polysomal high salt washes, which are the starting fraction for eukaryotic protein synthesis initiation factors, prior to further purification steps. The great advantage of the DC2 is that the processes of dialysis and concentration are simultaneous, efficient, gentle, and very fast.

CRC Labwasher: Although the Media Kitchen at Irvine washes glass- and plasticware on a recharge basis, the rates are high (and getting higher every year), the losses due to breakage and theft are excessive, and, due to inefficiency, approximately ¼ of the laboratory glass- and plastic-ware is out of use while it is being processed by the Media Kichen. My laboratory operates throughout weekends and holidays, but the Media Kitchen does not. This results in an unacceptable pileup of dirty glass-

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and plasticware during such periods. I have conducted a cost analysis covering a 2 year period, and find that the use of a laboratory dish washer, which we can operate ourselves, actually would save money after a period of use of between 7 to 8 months. In addition, the glass- and plasticware would be cleaner (student help in the Media Kitchen is rather casual and we have been troubled by glass- and plasticware returned in an obviously dirty and unacceptable condition, and by residual detergents on glassware used for tissue culture). Washing our own glass- and plasticware would effectively increase the laboratory supply by a significant fraction, since there would be no pileups of dirty glass- and plasticware. We have already switched from the Media Kitchen to our own apparatus for the washing of pipetts; the results have been entirely satisfactory. Radioactively contaminated glass- and plasticware will continue to be processed by the Media Kitchen, since they have special equipment for the handling of such items.

New Brunswick T-41 roller bottle apparatus for tissue culture: At the present time all of our tissue culture work is being done in glass and plastic flasks. For the large scale growth and production of AMV and of AMV-producing cells, a larger capacity system is required. The roller bottle apparatus is the most efficient means to this end.

Two New Brunswick CO-20 CO_2 incubators: As stated above, our tissue culture work is now performed in flasks which are purged with air containing 5% CO_2, sealed, and incubated at 37° in an incubator with a normal atmosphere. For plaque assays in dishes with loose covers, which must be removed periodically for observation, and then returned for further incubation, and for the establishment of primary and secondary cultures of chick embryo and bone marrow cells, the present arrangement is clearly unsatisfactory for work at 2 temperatures: 2 separate incubators are required.

Spinco SW-40-Ti rotor: This rotor centrifuges tubes 3 3/4 inches in length and offers the highest resolution available for zone velocity analyses through preformed sucrose density gradients. It is required for the analysis of the RNA prepared from AMV, for the separation of the high molecular weight 70S AMV RNA from the endogenous lower molecular weight RNA species, for the analysis of high molecular weight 70S AMV RNA purified by adsorption to, and elution from, microcrystalline cellulose, for the analysis of 70S AMV RNA before and after denaturation, and for the analyses of radioactive AMV RNA bound to ribosomes and ribosomal subunits and of radioactive AMV RNA which has entered polysomes. The rotor is able to centrifuge 6 tubes, thus allowing the analysis of several samples at once. The high centrifugal forces developed shorten the duration of the experiment and analyses of high molecular weight RNA, which are particularly vulnerable to traces of RNAase, require the use of such a rotor.

Second year -- Sorvall RC2-B centrifuge with SS-34, GSA, and GS-3 rotors: The 2 departmental RC2-Bs have suffered the same fate described for the departmental scintillation counters discussed above. The SS-34 rotor holds 50 ml tubes, the GSA rotor 250 ml bottles, and the GS-3 rotor 500 ml bottles. This centrifuge, noted for its reliability, and the 3 rotors are requested for the harvest of large quantities of virus, for the separation of phases during the phenol extraction of AMV RNA, for the harvest of myeloblasts and other cells from chickens and from tissue culture, and for the clarification of cell extracts. This piece

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of equipment is postponed until the second year because quantities of AMV, myeloblasts and other cells large enough to require its routine use are not expected to be ready until after the first year, although it would be very useful immediately during the phenol extraction of the RNA of AMV.

Third year -- VW&R vacuum pump: The vacuum pump in the laboratory lyophilizer was purchased used and it has been under constant use for 4 years. The last time that it was rebuilt and serviced, I was told that its life expectancy was no more than another 2 or 3 years. The new pump will replace the present pump in the lyophilizer.

Amicon MMC concentrator: Although this 8-place concentrator could be used immediately, its purchase has been set off until the third year. This piece of apparatus is designed to remove sucrose and concentrate samples recovered from sucrose gradients. It would be especially useful for the preparation of AMV RNA samples of varying sedimentation coefficient. It is fast in operation and this property is important during the processing of high molecular weight RNA. The samples that it accepts are small (10 ml); its use would be highly preferable to the usual practice of dialysis followed by ethanol precipitation.

Barnstead PM-70CB conductivity meter plus cell: This meter will be used to monitor the salt concentration during gradient elution of viral components from columns of ion exchangers, during gradient elution of viral RNA from microcrystalline cellulose, and for similar applications. It will also be used to check the concentrations of various reagent stock solutions. This instrument also would be useful immediately.

Spinco type 75 rotor: This angle rotor develops sufficient centrifugal force to sediment the 70S RNA from AMV in about 1 hour. It is therefore very useful for the recovery of such RNA from various solutions. The department has 1 65 rotor, but it is nearing the time when it must be derated. The purchase of this rotor has therefore been postponed until the third year.

New Brunswick G76 Gyratory water bath shaker with 3 platforms: This piece of apparatus, noted for its reliability, is requested for the incubation of tissue culture flasks during short term labeling experiments of AMV producing-myeloblasts and for the incubation of yeast cultures from which L-[^{35}S]methionine of very high specific activity is produced. This instrument could also be useful immediately.

Kelvinator -96° chest freezer UC-540: We presently have a Kelvinator upright -70° freezer. Although it is defrosted, cleaned, and materials no longer useful discarded, it has been in use for 4 years and its capacity has been reached. The chest freezer is requested for long term storage so that the upright freezer can be used for shorter term storage. This freezer could be put into service immediately.

Justification for consumable supplies:

The expenditures for consumable supplies are mostly self-explanatory. However 2 items might be worthy of some explanation.

Replacement drive for the Spinco L3-50: These drives are requested every 2 years; that is the average life-time of the L3-50 drive. Without such replacement drives, the L3-50 (purchased with A. C. S. funds from an earlier grant) would become useless.

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Animals and animal boarding in the U. C. I. Vivarium: The actual purchase price of animals is but a small % of this item. Vivarium costs at U. C. I. (animals must be kept within the Vivarium; there is no alternative) are extremely high; over the past few years they have increased dramatically, and they undoubtedly will go higher yet. Arguments with the Vivarium personnel and with the administration have come to naught.

Economies made in the area of consumable supplies:

Care of eggs, chicks, and chickens: Laboratory personnel care entirely for the eggs and these animals; we purchase the feed and the bedding, clean the incubator and brooder (which we originally purchased), and clean the room in which they are kept. The Vivarium has the grace to only charge us half price for the maintenance of the animals under these conditions. At least we save half.

tRNA: We isolate our own tRNA from chicken liver, chicken reticulocytes, rabbit liver, rabbit reticulocytes, rat liver, and chick embryos and myeloblasts. It is true that it is cheaper than commercial materials, but the main reason is that our product is much purier and more active.

L-[^{35}S]Methionine: We produce this material in the laboratory because it is much less expensive than the commercial products (1/50), but the main reason is the same one as given above; the product is isotopically pure -- commercial samples are grossly contaminated with methionine sulfoxide and several unknown sulfur-containing compounds.

Consultants in the area of the project proposal:

Dr. Gale Granger will offer advice and counsel in the area of immunology; this is his field of expertise. He is in the same department as I.

Dr. Charles Gordon will offer his services, advice and counsel in the area of electron microscopy; this is his field of expertise. He is in the same department as I.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
In Vitro Translation of Oncogenic C-Type Viral RNA	Cancer Research Coordinating Committee of the University of Calif. UCI-1719	12,000	7/1/73-6/30/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Temperature Sensitive and Conditional Lethal Mutants of Avian Myeloblastosis Virus	Calif. Div. of the ACS UCI-1723 Grant deferred -- Agency has no available funds.	30,364	9/1/73-8/31/74
Isolation and Characterization of Temperature Sensitive and Conditional Lethal Mutants of Avian Myeloblastosis Virus and the Identification of Virus-Specific Products Synthesized in Vitro under the Direction of Wild-Type and Mutant Viral RNA	Am. Cancer Society UCI-1730 Grant deferred -- Insufficient funds at present -- will be considered later for possible funding as of 7/1/74	167,256	1/1/74-12/31/76
Studies on Chicken Leukemia Viruses	Damon Runyon Mem. Fund This grant seeks support for graduate students and for postdoctoral students.	98,668	9/1/73-8/31/75
Biochemical and Genetic Studies on AMV	Jane Coffin Childs Mem. Fund This grant asks support for grad. & postdoctoral students.	63,982	9/1/73-8/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator Wendell M Stanley Jr

Typed Name Wendell M. Stanley, Jr.

Signature Wendell M Stanley Jr Date July 31, 197

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. L. E. Cox, Vice Chancellor-Business & Finance

Mailing address for checks

Responsible officer of institution

Typed Name

Title

Signature Date

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1003544199

Source: https://www.industrydocuments.ucsf.edu/docs/fgdm0000

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